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Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-116536>

Conference or Workshop Item

Accepted Version

Originally published at:

Favrot, C (2015). Polymerase chain reaction: advantages and drawbacks. In: 3. Congresso Latinoamericano de Dermatologia Veterinaria, Buenos Aires, Argentina, 26 November 2015 - 27 November 2015, s.n..

POLYMERASE CHAIN REACTION: ADVANTAGES AND DRAWBACKS

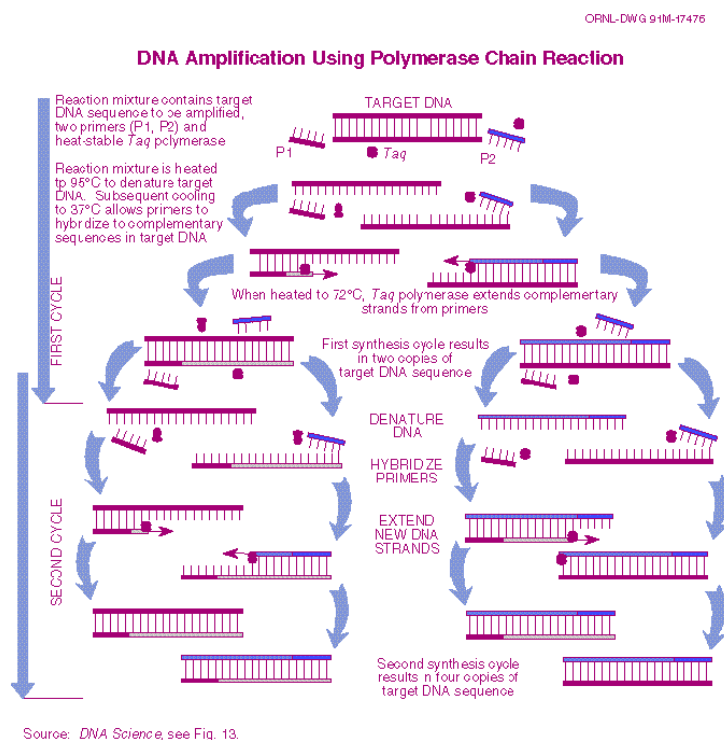
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To confirm or to rule out the presence of one specific pathogenic agent in a tissue sample is the most common application of polymerase chain reaction (PCR).

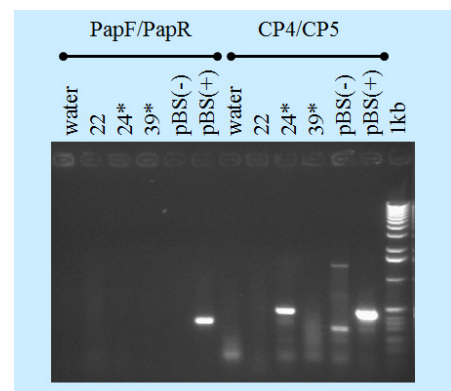
BASICS TO GET STARTED:

PCR uses short segments of nucleotides, called primers. The sequences of these primers are complementary to the sequences of the target (microbe nucleic acids). The primers serve as templates upon which a new DNA molecule can be



complementary regions on the target DNA. The solution is subsequently heated again to promote DNA extension (addition of nucleotides to the primers ends). The product is consequently a sequence of DNA delimited by the two primers and

synthesized. They may be of variable length but are usually 15-30bp long. The primers are added to a solution of containing a DNA polymerase, each of the four nucleotides and a source of DNA containing the target sequence. The solution is heated to denature the DNA (separate into its two strands), then cooled to let primers bind to



strictly identical to the target DNA. The solution will be submitted to 25 to 40 cycles (amplification). The whole reaction results in an accumulation of millions of copies of the target DNA. Confirmation of the correctly sized amplified DNA product is usually carried out by agarose gel electrophoresis. The picture on the right is one typical example of the end-point PCR: two different sets of primers were used (PAPF and CP4) and three samples were tested (22,24,39). With first set of primers, all samples were negative while, with the second one, sample 24 was positive.

MATERIAL

PCR is a powerful tool to amplify minute amounts of nucleic acids. Due to its unprecedented sensitivity, the method has become an essential diagnostic and research tool for infectious dermatology. Additionally, PCR can be used on all tissues or samples (fresh tissues, paraffin-embedded tissues, blood, feces etc...). It is also possible to analyze samples of poor conditions because only relatively short intact sequences of DNA are required. Archival materials can consequently be used for retrospective studies. In this latter case, however, amplification depends on the conservation of the target DNA. It has, for example, been demonstrated that DNA could be spoiled by long-stay in formalin and that amplification subsequently failed.

PCR HAS SOME LIMITATIONS....

PCR is not only a very sensitive technique but also a very specific one: The primers are usually directly complementary to the target sequence and DNA can only be amplified if the matching target DNA-primer is adequate. In fact, as primers are directly complementary to target DNA, amplification only occurs if the target is exactly or closely related to the DNA sequence of the expected causative agent. For example, primers designed to amplify canine oral papillomavirus DNA will probably not amplify the DNA of another canine papillomavirus. PCR demands that sequence information be available for at least a part of the DNA that is to be amplified. Interpreting the clinical relevance of a positive PCR amplification can also be challenging. In fact some extremely sensitive nested PCR detect even 0.05 viral copy per cell. The presence of such trace amounts of DNA does not indicate a

productive infection but probably a latent or clinically non-relevant infection. For this reason, one must interpret cautiously some PCR results.

Last but not least, PCR does not allow localization of the nucleic acids. It is consequently not possible to differentiate a clinical infection from a contamination. It has, for example been shown, that papillomavirus DNA can be amplified from virtually each sample of normal human skin. But the detection rate is much lower when the *stratum corneum* of these samples is removed. This indicates that most of these papillomavirus are probably contaminant.

BUT IT IS NOT THAT BAD: THERE IS ALWAYS A SOLUTION!

PCR cannot amplify RNA...

As PCR cannot amplify RNA, detection of RNA is carried out through **reverse-transcriptase PCR (RT-PCR)**. The enzyme reverse transcriptase allows synthesis of cDNA from a RNA template. PCR is subsequently carried out, using cDNA as target DNA.

PCR only amplifies specific targets...

To circumvent this drawback **degenerate or consensus primers** can be designed: Such primers allow the amplification of nucleic acids of multiple related infectious agents (Some degenerated primers are able to amplify nucleic acids of up to 70 human papillomaviruses, for example).

PCR amplifies only a very limited part of the genome...

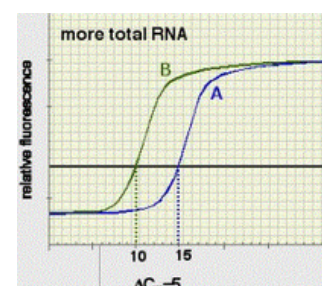
That is true but it is also possible, in some instances, to carry out the reaction with several set of primers: **Multiplex PCR** consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

PCR may sometimes not be specific enough...

That is the reason why **nested PCR** were developed! In fact, nested PCR increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

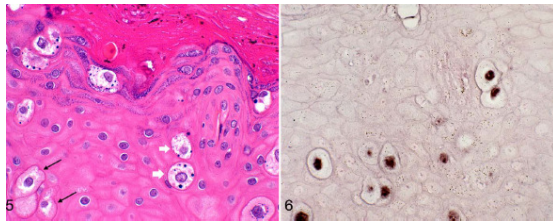
PCR is not quantitative...

But it can be... **Real-time PCR (qPCR; RT should be reserved for Reverse Transcriptase and Real Time should be noted Q for quantitative!)** has been developed to estimate nucleic acids concentration within the samples. qPCR is used to measure the quantity of a target sequence (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Quantitative PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. *Quantitative PCR* has a very high degree of precision. Quantitative PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. In the last few years, in example, such procedure were used in two different studies to assess the expression of thymic stromal lymphopoietin (TSLP) and fillagrin, respectively, in skin samples retrieved from dogs with atopic dermatitis. For such studies a references gene should be used, whose expression is stable and consistent in canine skin. In the picture on the right, one should imagine that A is the curve of the reference gene and B the curve of the studied genes: as the exponential phase of the reaction begin with less cycles (in this case, n:10) than for the reference gene (n:15), we can conclude that the studied gene is expressed more than the reference gene. If the expression of the reference gene is known, expression of the studied can be computed.



It is not possible to know where the DNA was in the sample...

In situ hybridization can be regarded as a combination of PCR and immunochemistry and allows cellular localization of the target DNA. Basically, PCR



is used to generate dye-associated primers that will serve subsequently for the immunochemistry. We have used this tool to demonstrate that viral DNA was present in

the koilocytes of early stages of penis carcinomas in horses. On the left part of the picture, we can see the koilocytes and on the right part, we see that these koilocytes harbor the viral DNA.

PCR FROM BENCH TO BEDSIDE: WONDERFUL BUT.... BE CAREFUL!

As mentioned above, PCR is a wonderful tool for research but also for daily diagnosis. One should however be aware of one possible pitfall: the sensitivity of the method. As already said, some very sensitive PCR settings may be able to identify as few as one genome copy within 100 cells! It is very unlikely that these few microorganisms may cause any damage to the host but... the test is positive! One should consequently interpret PCR results cautiously. Exactly like the IgE serology tests that identify sensitization (and not allergy!), PCR identifies infection, but does not say anything on the actual link between this infection and the clinical signs. Let's take the example of leishmaniasis!

Before 2000, most authors agreed that the prevalence of the disease was from 1 to 15% in endemic areas of Europe. But, as soon as PCR studies began, data changed. It was suddenly obvious that the great majority, if not all, dogs living in endemic areas were infected. But only some of them were seropositive and few presented with clinical signs! That is the reason why PCR is NOT a good test to assess whether leishmania infection explain the clinical signs of one specific dog. The gold standard to answer this question remains the detection of leishmania-specific IgG!

Selected References:[1, 2]

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2. Sellon, R.K., *Update on molecular techniques for diagnostic testing of infectious disease.* Vet Clin North Am Small Anim Pract, 2003. **33**(4): p. 677-93.